Kidney International, Vol. 44 (1993), pp. 557–564

Glomerular fibrinolytic activity in anti-GBM glomerulonephritis in rabbits

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Glomerular fibrinolytic activity in anti-GBM glomerulonephritis in rabbits. Fibrin is an important mediator of injury in severe proliferative forms of glomerulonephritis (GN). Normal glomeruli express fibrinolytic activity, which may protect against the injurious effects of fibrin deposition. Changes in glomerular fibrinolytic activity (GFA) may play an important role in modulating fibrin accumulation in GN. To study the changes in GFA associated with fibrin deposition in GN, autologous phase anti-glomerular basement antibody initiated GN (anti-GBM GN) was studied in rabbits. Net GFA was significantly reduced in association with glomerular fibrin deposition (1.3 ± 0.8 ng fibrin lysed/10^3 glomeruli/2 hr, normal 57.1 ± 25.4 ng fibrin lysed/10^3 glomeruli/2 hr, $P < 0.02$). Reduced GFA in fibrin associated GN was associated with decreased expression of tissue type plasminogen activator (tPA) and increased expression of plasminogen activator inhibitor type-1 (PAI-1) and glomerular macrophage infiltration. In a fibrin independent model of anti-GBM induced GN (heterologous phase), with equivalent injury (proteinuria), net GFA was increased (174 ± 64 ng fibrin lysed/10^3 glomeruli/2 hr). This was associated with increased tPA and uPA, and decreased PAI-1 in the absence of significant macrophage infiltration. These studies demonstrate that fibrin deposition in GN is associated with a net reduction of GFA, attributable to reduced expression of plasminogen activators and augmentation of PAI-1. Reduction of GFA may potentiate glomerular fibrin deposition and consequent glomerular injury. The association between glomerular macrophage influx and reduction in GFA suggests that this change may be directed by macrophages.

Fibrin is a prominent glomerular participant in aggressive proliferative and crescentic human glomerulonephritis (GN) [1–3]. In experimental GN, induced by either chronic immune complex disease [4] or anti-GBM antibodies [5], glomerular injury is similarly associated with crescentic GN and renal failure. The important role played by glomerular fibrin deposition has been best demonstrated by observing the beneficial effects associated with defibrination in these rabbit models of GN where crescent formation and renal failure were substantially prevented [4, 5].

Recent studies have suggested an important role for macrophages as initiators of glomerular fibrin deposition in GN by their enhanced expression of tissue factor. In patients with crescentic GN, a close association has been observed between glomerular injury, fibrin deposition, macrophage accumulation and increased tissue factor expression [6]. Isolation of glomeruli from patients with these glomerular findings have shown vastly augmented expression of glomerular procoagulant activity (PCA) [7]. Similar observations have been made in experimental crescentic GN in rabbits induced by anti-GBM antibodies (autologous phase). Glomerular injury in this situation is associated with glomerular influx of macrophages and their local activation to enhance PCA expression [8–10]. This is closely associated with the initiation of glomerular fibrin deposition. Macrophage depletion and repletion studies confirm the important role of infiltrating macrophages in glomerular procoagulant enhancement and fibrin deposition [10]. Thus, this model demonstrates very similar events to those occurring in human crescentic GN and studies of the mechanisms of fibrin deposition are likely to be relevant to humans.

The role of fibrinolytic molecules in fibrin deposition in GN is not clear. The potential for the fibrinolytic system to play a protective role is again highlighted by studies in anti-GBM GN in rabbits showing benefit from treatment with pharmacological doses of tissue plasminogen activator [11] or streptokinase [12]. Use of antifibrinolytic agents, both in humans [13] and experimental GN [14], has been demonstrated to enhance glomerular fibrin deposition.

We have therefore sought to characterize the presence and nature of glomerular fibrinolytic activity (GFA) in normal rabbit glomeruli, and to study the changes observed in the relevant and otherwise well-characterized model of anti-GBM antibody-induced crescentic GN in rabbits.

Methods

Animals

Male New Zealand White rabbits weighing 1.8 to 2.5 kg were used in these experiments.

Preparation of heterologous anti-GBM globulin

Horse anti-rabbit GBM antibody was prepared by repeated immunization of a horse with purified particulate rabbit GBM in

Received for publication November 23, 1992
and in revised form April 26, 1993
Accepted for publication April 26, 1993

phosphate buffered saline (PBS). The horse serum was extensively absorbed against rabbit platelets, leukocytes and erythrocytes, and a globulin fraction was prepared by precipitation with ammonium sulphate at a final concentration of 50%.

Experimental models

Two models of anti-GBM antibody initiated GN were studied.

Autologous phase anti-GBM antibody initiated GN. Autologous phase anti-GBM GN was induced by intravenous administration of horse anti-rabbit GBM globulin (25 mg/kg) to rabbits presensitized to horse globulin five days earlier by subcutaneous injection of horse globulin (4 mg) in Freund's complete adjuvant (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). This dose of anti-GBM globulin resulted in a mean binding of 28.1 ± 2.7 μg of specific kidney fixing horse globulin per gram wet weight of kidney. Injury was assessed 96 hours after administration of anti-GBM globulin. Defibrination studies have previously demonstrated that the development of progressive renal impairment and crescents in this model is fibrin dependent [5, 12, 15].

Heterologous phase anti-GBM antibody initiated GN. Heterologous phase anti-GBM GN was initiated in non-sensitized rabbits by an intravenous dose of horse anti-GBM globulin (45 mg/kg) which bound 40.4 ± 1.8 μg of specific kidney fixing horse globulin per gram wet weight of kidney, and produced immediate proteinuria. Injury in this model was assessed after 24 hours. In this model, significant glomerular fibrin deposition does not occur [10].

Isolation of glomeruli

Renal cortical tissue was collected aseptically into culture medium (Eagles Minimal Essential Medium, Commonwealth Serum Laboratories) containing 10% fetal calf serum (FCS, Flow Laboratories, North Ryde, NSW, Australia) for preparation of glomerular conditioned medium or into ice-cold 20 mM Tris-HCl buffer (pH 7.4 containing 125 mM NaCl, 10 mM KCl, 10 mM sodium acetate and 5 mM glucose) for preparation of glomerular lysates.

Preparation of glomerular conditioned medium (supernatants)

Glomeruli were isolated under sterile conditions by a graded sieving technique [16] to obtain approximately 10^5 glomeruli. Contamination with tubular fragments was less than 5% (as assessed by phase contrast microscopy). Glomeruli were cultured in 75 cm^2 polystyrene tissue culture flasks (Sterilin, Feltham, UK) for 16 hours at 37°C in a 5% CO_2/air humidified atmosphere, at a concentration of 5×10^3 glomeruli per ml in serum-supplemented medium. Conditioned medium was collected and centrifuged at 1600 g for five minutes to remove glomeruli and the supernatant stored at −70°C.

Preparation of glomerular lysates

Glomeruli were washed and resuspended at a concentration of 5×10^3/ml in Tris-HCl buffer containing 1 mM $CaCl_2$ and incubated for 30 minutes at 37°C. Glomerular lysates were prepared by solubilization in 0.1% Triton X-100 at room temperature for seven minutes, collected after centrifugation at 3000 g for 10 minutes at 4°C and stored at −70°C.

Assessment of glomerular macrophage infiltration

Glomerular macrophages numbers were determined by individual droplet culture as previously described [17] and by immunohistochemical staining with a monoclonal anti-rabbit macrophage antibody [18] (RAM-11, a gift from Dr. A. Gown, Seattle, Washington, USA).

Assessment of glomerular injury

Proteinuria. Rabbits were housed in metabolic cages to collect their urine output over the final 24 hours of each experiment. Protein concentrations were determined by the Bradford method [19]. Absorbance of light (595 nm wavelength) was measured 10 minutes after the addition of 3 ml of 100 μg/ml Electran Page Blue G90 (BDH Chemicals, Poole, UK) in 5% ethanol/8.1% orthophosphoric acid to 20 μl of each urine sample. Values were calculated from a standard curve derived from bovine serum albumin, (BSA, Miles Inc., Kankakee, Illinois, USA).

Histology. Kidney tissue was fixed in Bouin's fixative and stained with periodic acid-Schiff reagent to assess light microscopic appearances. Tissue for immunofluorescence (IF) was frozen in liquid nitrogen, then stored at −70°C. Six micrometer cryostat sections were stained with goat anti-rabbit fibrinogen (Research Plus Laboratories, Bayonne, New Jersey, USA), to assess glomerular fibrin deposition.

Measurement of net glomerular fibrinolytic activity

A solid phase ^{125}I fibrin lysis assay based on the assay of Moroz and Gilmore [20] was used to measure the net plasminogen dependent GFA.

Rabbit fibrinogen (Sigma Chemical Company, St. Louis, Missouri, USA) was radiolabeled by a modified chloramine T method and extensively dialyzed against PBS. The ^{125}I activity was determined to be 97% protein bound after precipitation with trichloroacetic acid, and more than 80% of the radiolabeled fibrinogen was clottable upon the addition of thrombin (Armour Pharmaceutical Company, Kankakee, Illinois, USA). Trace labeled fibrinogen (0.5 mg/ml in 0.015 M phosphate buffer, pH 8.2) of known specific activity was absorbed to 96-well U bottom polystyrene plates (Disposable Products, Adelaide, Australia) by incubation for three hours at 37°C. The plates were washed three times in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.1 M NaCl) and treated with thrombin (10 U/ml in 0.16 M NaCl) for 15 minutes at 37°C to generate a fibrin matrix. After three washings, the plates were stored at 4°C for a maximum of seven days with 0.05 M Tris HCl buffer. The mean binding of fibrinogen to the plates was 1.8 ± 0.25 μg/well.

Glomerular supernatants (60 μl) were incubated in fibrin coated wells, for two hours at 37°C in the presence or absence of rabbit plasminogen (0.025 casein units, Sigma) as previously described [21]. The ^{125}I activity released into the soluble phase was used to determine the quantity of fibrin lysed from the specific activity of the clottable fibrinogen used to coat the plates. The difference between fibrin lysis in the presence and absence of plasminogen was used to determine the plasminogen dependent fibrinolytic activity of the glomerular supernatants, and the results expressed as ng fibrin lysed per 10^3 glomeruli per two hours (ng fibrin/10^3 gloms/2 hr). All samples were assayed in triplicate.

Detection and identification of plasminogen activators by fibrin autography

Fibrin autography, performed using standard techniques [22], was used to detect plasminogen activator activity in glomerular supernatants and lysates. Supernatants, human urokinase plasminogen activator (uPA; American Diagnostica, Greenwich, Connecticut, USA) and human tissue plasminogen activator (tPA; American Diagnostica) were run in parallel lanes on a 10% SDS polyacrylamide gel, which was then overlaid on a fibrin agar indicator gel prepared using plasminogen-rich bovine fibrinogen (Sigma) and bovine thrombin (Armour). The gels were incubated at 37°C in a humidified atmosphere to reveal lysis zones corresponding to the presence of plasminogen activator activity. There was no detectable lytic activity in serum-supplemented culture medium alone. Equal volumes of supernatants from glomeruli cultured at a standard concentration (5×10^3/ml) were added in each lane. Glomerular lysates and standards were run on separate gels.

Pre-absorption of glomerular supernatants and lysates with antisera to uPA and tPA was used to determine the antigenic nature of the plasminogen activators. Supernatants were incubated with goat anti-human uPA antibody (American Diagnostica), goat anti-human tPA antibody (American Diagnostica) or non-immune goat serum bound to CNBr-activated sepharose for 24 hours at 4°C. The sepharose beads were removed by centrifugation and the plasminogen activator activity remaining was then analyzed by fibrin autography as described.

Quantitation of tPA functional activity

Quantitation of tPA activity in glomerular supernatants and lysates was performed according to a previously described method [23, 24]. The high affinity of tPA for a fibrin matrix bound to polyvinyl chloride microtiter plates was used to separate plasminogen activator activity of tPA from uPA. Using this technique, 90 ± 2% of the activity of pure human tPA and 18 ± 2% of pure human uPA bound to the fibrin matrix. Conversion of plasminogen to plasmin was detected by the generation of a colored product from a plasmin specific chromogenic substrate (H-D-Nle-HHT-Lys-pNA.2 AcOH, American Diagnostica).

Glomerular supernatants and lysates were diluted with an equal volume of PBS containing 4 mg/ml BSA and 0.01% Tween 20 (PBT buffer). tPA standards were also prepared in serum-supplemented culture medium or Tris lysate buffer and diluted in PBT buffer. Both tPA standards and samples (100 μl) were incubated for 75 minutes at 37°C in fibrin coated wells. The wells were washed three times with PBT buffer, and then incubated for four hours at 37°C with human glu-type plasminogen (0.09 mg/ml final concentration, Technoclone, Vienna, Austria) and chromogenic plasmin substrate at a final concentration of 0.9 mM in 100 μl of PBT buffer. The reaction product (paranitroanalide) was detected by its absorption at 405 nm using a micro-ELISA reader (Titertek Multiskan; Flow Laboratories). Plates were blanked on a well containing Eagles medium with 10% FCS or Tris lysate buffer. All standards and samples were assayed in duplicate. The lower limit of detection of tPA was less than 0.01 international units/ml (IU/ml). tPA activity of the standards was calculated from the concentration using a conversion factor of 1 IU/ml being equivalent to 2 ng/ml tPA.

Quantitation of uPA functional activity

uPA activity was measured in a functional assay using a uPA specific chromogenic substrate (Spectrozyme 221, American Diagnostica). Fifty microliters of glomerular supernatant, lysate or purified human uPA (Ukidan, Serono, Rome, Italy) diluted with an equal volume of 50 mM Tris-HCl buffer containing 0.1% BSA (pH 7.5) was incubated for three hours with 100 μl of 0.5 mM substrate in a microtiter plate at room temperature. The colored product was measured in an ELISA plate reader by its absorbance at 405 nm. All samples and standards were assayed in duplicate. Standards were diluted in serum-supplemented Eagles medium or Tris lysate buffer to provide separate reference curves for glomerular supernatants and lysates. Activity/concentration curves were linear in the range 0.2 to 30 IU/ml.

Measurement of tPA antigen

tPA antigen in glomerular supernatants and lysates was measured by ELISA (Imubind tPA, American Diagnostica). This assay employs a sandwich technique using a polyclonal anti-human tPA antibody which cross reacts with rabbit tPA. The assay detects both free tPA and tPA complexed to PAI and has a lower detection limit of 0.1 ng/ml. Human single chain tPA was used as a standard in this assay. All standards and samples were assayed in duplicate.

Detection of plasminogen activator inhibitor by reverse fibrin autography

The capacity of glomerular supernatants and lysates to inhibit uPA initiated lysis of a fibrin agar gel was used to detect PAI activity as described previously [25]. Glomerular supernatants and recombinant human plasminogen activator inhibitor type 1 (rhPAI-1) (Dr. P. DeClerck, Leuven, Belgium) were run in parallel lanes in 10% SDS polyacrylamide gel, and then overlaid on a freshly prepared fibrin indicator gel containing uPA (0.2 IU/ml). The gels were incubated at 37°C in a humid atmosphere, and bands of fibrin resistant to lysis indicated PAI activity. Culture medium (containing serum) did not show areas of lysis inhibiting activity.

Measurement of PAI activity

PAI-1 activity was measured using a functional assay by its capacity to inhibit uPA dependent cleavage of a chromogenic substrate (Spectrozyme 221) based on a previously described method [26]. Glomerular supernatants, lysates and human cell line derived PAI-1 (American Diagnostica) in culture medium or lysate buffer, were activated by dialysis against 4 M guanidine HCl for four hours at 37°C, and were then dialyzed for 16 hours at 4°C against PBS with 0.01% Tween-80 [27]. uPA (Serono) was diluted to 4 IU/ml in "activity buffer" containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% BSA and 0.01% Tween-80. Samples or PAI-1 standards (50 μl) were incubated with uPA (50 μl) in microtiter plates for 20 minutes at 37°C. Chromogenic substrate (100 μl of 0.5 mM) was subsequently added and the generation of colored product was determined by the absorption at 405 nm after four hours at room temperature. All standards and samples were run in triplicate.

Table 1. Proteinuria, glomerular macrophages, glomerular fibrin deposition (GFD) and glomerular fibrinolytic activity (GFA), in normal rabbits and rabbits with anti-GBM GN

	Proteinuria *mg/24 hr*	Glomerular macrophages *mac/glom*	GFD (0 to +++)	GFA *ng fibrin/ 10^3 glom/ 2 hr*
Normal	14.9 ± 2.0	0.3 ± 0.2	0	57.1 ± 25.4
Fibrin associated GN	1549 ± 262	63.6 ± 5.8	+++	1.3 ± 0.8[a]
Fibrin independent GN	1567 ± 351	0.5 ± 0.3	0	174 ± 64

[a] $P < 0.02$ c.f. Normal

Activated human PAI-1 produced a dose dependent inhibition of uPA with a linear relationship on semilog scale in the range of 1 to 100 ng/ml PAI-1 (correlation coefficient 0.98). PAI activity was calculated from the difference between uPA activity in culture medium or lysate buffer and the uPA activity in samples, by reference to a standard curve of uPA activity. PAI activity is expressed as arbitrary units (aU/10^5 glom) where 1 aU represents the inhibition of 1 IU of uPA activity. In this assay, 10 ng/ml PAI-1 standard in culture medium exhibited 1.28 IU uPA inhibiting activity.

Experimental design and statistics

The following groups of rabbits were studied: (i) normal rabbits ($N = 6$); (ii) rabbits with autologous phase anti-GBM GN on day 4 of disease ($N = 6$); and (iii) rabbits with heterologous phase anti-GBM GN ($N = 6$).

Results are expressed as the mean ± the standard error of the mean (SEM) and statistical significance assessed by a two-tailed Mann Whitney U test.

Results

Autologous phase anti-GBM GN

Rabbits with autologous phase anti-GBM GN (fibrin associated GN) developed significant proteinuria (1549 ± 262 mg/24 hr, normal 14.8 ± 2.0; $P < 0.005$) in association with macrophage infiltration (63.6 ± 5.8 mac/glom, normal 0.3 ± 0.2 mac/glom) and a proliferative GN with prominent glomerular fibrin deposition and fibrinous crescents (Table 1).

Heterologous phase anti-GBM GN

Rabbits with heterologous phase injury (fibrin independent GN) developed similar proteinuria (1567 ± 351 mg/24 hr) to rabbits with fibrin-associated autologous phase anti-GBM GN. Their glomerular lesion was characterized by a proliferative GN, with prominent neutrophil infiltration, in the absence of glomerular fibrin deposition. Glomerular macrophage numbers (0.5 ± 0.3 mac/glom) were not different to macrophage numbers in normal glomeruli (Table 1).

Net glomerular plasminogen-dependent fibrinolytic activity (GFA) in GN

GFA in glomerular supernatants from rabbits with fibrin associated GN (autologous phase anti-GBM GN) was significantly reduced (1.3 ± 0.8 ng fibrin/10^3 glom/2 hr) compared to supernatants from normal glomeruli (57.1 ± 25.4 ng fibrin/10^3 glom/2 hr; $P < 0.02$; Table 1). In glomerular supernatants from rabbits with fibrin independent GN (heterologous phase anti-GBM GN), GFA was increased compared to normal 174 ± 64 ng fibrin/10^3 glom/2 hr).

Demonstration and characterization of glomerular plasminogen activator activity by fibrin autography

Glomerular supernatants from normal rabbits and rabbits with fibrin associated and fibrin independent GN showed two bands of fibrinolytic activity with molecular weights of 110 and 120 kD, suggesting plasminogen activator-plasminogen activator inhibitor complexes (Fig. 1). Glomerular supernatants from rabbits with fibrin associated GN produced smaller areas of lysis (lane B1) than that produced by the same amount of glomerular supernatant from normal rabbits (lane A1) or rabbits with fibrin independent GN (lane C1). Fibrinolytic activity in each group was substantially removed by absorption of glomerular supernatants with anti-tPA antibody (lanes A2, B2, C2) and to a lesser extent by absorption with anti-uPA antibody (lanes A3, B3, C3).

Glomerular lysates in each of the three groups showed a single area of lytic activity with a molecular weight of 45 kD (Fig. 2). Lysates from glomeruli of rabbits with fibrin associated GN (lane F1) produced a smaller area of lysis than glomerular lysates from normal rabbits (lanes D1) and rabbits with fibrin independent GN (lanes E1). Absorption of lysates with anti-uPA antibody completely removed the lytic activity (lanes D3, E3, F3). Absorption with anti-tPA antibody did not affect the area of lysis activity (lanes D2, E2, F2).

Quantitation of tPA and uPA activity

The predominant plasminogen activator activity in glomerular supernatants was tPA (Tables 2 and 3). Supernatants from normal glomeruli contained 8.7 ± 3.7 IU tPA activity/10^5 glom and 6.7 ± 1.3 IU uPA activity/10^5 glom. Glomerular supernatants from rabbits with fibrin associated GN contained less tPA activity (0.7 ± 0.5 IU/10^5 glom) than normal supernatants ($P < 0.08$). uPA activity (6.5 ± 1.8 IU/10^5 glom) was similar to normal. In fibrin independent GN, tPA activity in glomerular supernatants (12.9 ± 8.7 IU/10^5 glom) was not significantly different to normal. uPA activity in glomerular supernatants (16.8 ± 1.2 IU/10^5 glom) was significantly increased compared to normal ($P < 0.002$).

Glomerular lysates from all three groups contained substantial amounts of uPA activity. There was no significant change in the uPA activity in glomerular lysates from rabbits with fibrin independent GN (115 ± 21 IU/10^5 glom) and fibrin associated GN (95 ± 12 IU/10^5 glom) and normal rabbits 132 ± 14 IU/10^5 glom). tPA activity was undetectable in glomerular lysates in all groups.

Glomerular tPA antigen

Normal glomeruli contained 69.4 ± 22.4 ng tPA/10^5 glom in supernatants and 2.9 ± 1.9 ng tPA/10^5 glom in lysates. Supernatants from glomeruli of rabbits with fibrin associated GN showed a reduction in tPA antigen (17.7 ± 11.0 ng/10^5 glom) compared to supernatants from normal glomeruli ($P < 0.06$).

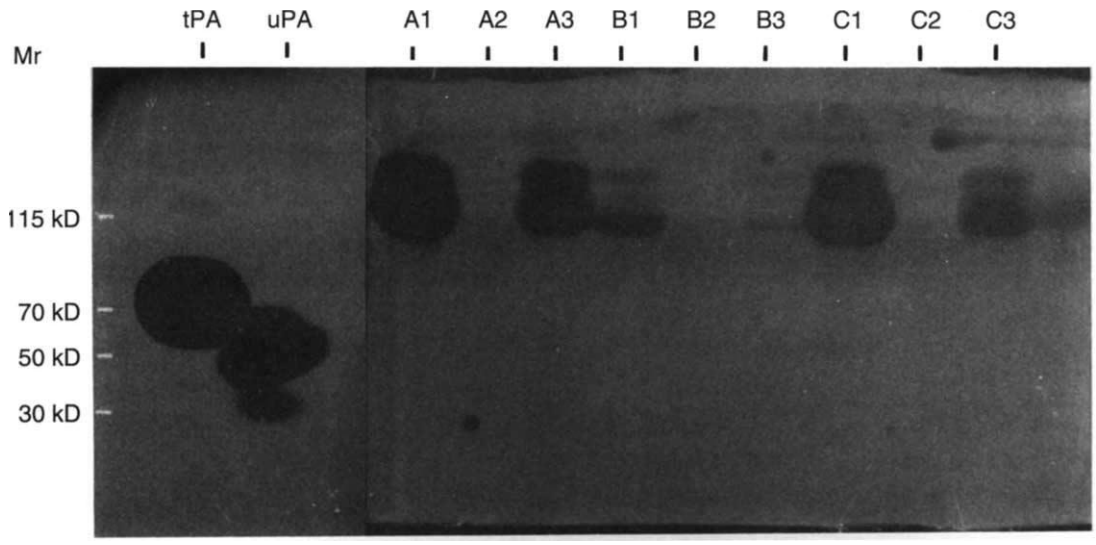

Fig. 1. *Plasminogen activator activity demonstrated by fibrin autography in glomerular supernatants, (preabsorbed with normal goat serum) from a normal rabbit (lane A1), a rabbit with autologous phase anti-GBM GN (lane B1) and a rabbit with heterologous phase anti-GBM GN (lane C1).* The same supernatants were preabsorbed with anti-tPA antibody (lanes A2, B2, C2) or anti-uPA antibody (lanes A3, B3, C3). Pure human tPA and uPA were run as standards. Molecular weight calibrations are indicated.

tPA antigen in glomerular lysates from these rabbits (1.7 ± 0.9 ng/10^5 glom) was not significantly reduced compared to normal. In fibrin independent GN, tPA was not significantly different from normal in glomerular supernatants (99.0 ± 22.3 ng/10^5 glom) or lysates (1.5 ± 0.9 ng/10^5 glom).

Demonstration of glomerular plasminogen activator inhibitor activity by reverse fibrin autography

Glomerular supernatants from normal rabbits exhibited an area of inhibitor activity similar to that seen with human recombinant PAI-1 (Fig. 3, left hand lane) with apparent molecular weight of 48 kD. Glomerular supernatants from rabbits with fibrin associated GN exhibited a marked increase in the area of inhibitor activity (lane 2) compared to supernatants of normal glomeruli (lane 1). Glomerular supernatants from rabbits with fibrin independent GN (lane 3) exhibited an area of inhibitor activity similar to normal glomeruli.

Glomerular PAI activity

Supernatants from normal glomeruli contained 9.6 ± 4.4 aU/10^5 glom of PAI activity (Table 4). Glomerular supernatants from rabbits with fibrin associated GN contained higher PAI activity (42.4 ± 11.2 aU/10^5 glom) than normal glomerular supernatants ($P < 0.03$). PAI activity was in glomerular supernatants from rabbits with fibrin independent GN (0.8 ± 0.8 aU/10^5 glom) was not significantly different from normals ($P = 0.12$). PAI activity was undetectable in glomerular lysates in all groups.

Discussion

Autologous phase anti-GBM GN provides a model of fibrin dependent GN which closely parallels crescentic human GN. These diseases exhibit similar pathological features including severe proliferative glomerular injury, macrophage infiltration, crescent formation and fibrin deposition associated with augmentation of glomerular tissue factor expression. In addition, normal rabbit and human glomeruli exhibit similar profiles of plasminogen activators and inhibitors [24, 28]. Thus, changes in these molecules in this rabbit model of GN are likely to be relevant to the human disease.

The current studies demonstrate a significant reduction in GFA in association with fibrin deposition in GN. The release of tPA and to a lesser extent uPA (demonstrated by fibrin autography) into the conditioned medium of cultured glomeruli was reduced. Urokinase activity, which was abundant in the membrane fractions of glomeruli, was unaffected suggesting that this pool of uPA may not have an important role in determining glomerular fibrin deposition. Augmentation of PAI-1 also contributed to the reduction of GFA. Recent reports suggest that up-regulation of PAI-1 may also contribute to glomerular fibrin deposition in human GN [29]. These changes in plasminogen activators and plasminogen activator inhibitors may act synergistically with the augmentation of procoagulant activity to enhance accumulation of fibrin in glomeruli and subsequent injury in GN. By contrast in fibrin independent GN, glomerular plasminogen activator production showed a tendency to increase and PAI-1 production was decreased.

A number of factors may contribute to the changes in expression of pro- and anti-fibrinolytic molecules in glomeruli of rabbits developing GN. The most striking among these appears to be the cellular mediators of injury involved, in particular, macrophages. In human GN, glomerular macrophage accumulation is associated with severe proliferative GN, fibrin deposition and crescent formation [30]. Similarly, in anti-GBM GN in rabbits, macrophage depletion prevents fibrin deposition, crescent formation and augmentation of glomerular procoagulant activity [10, 31]. The current studies demonstrate an association between macrophage infiltration and down-regulation of GFA, consistent with their role in initiating glomerular fibrin deposition. Earlier studies by Stark, Miller and Michael [32] suggest that similar down-regulation of GFA may occur in association with fibrin deposition in chronic immune complex GN in rabbits. A close correlation between

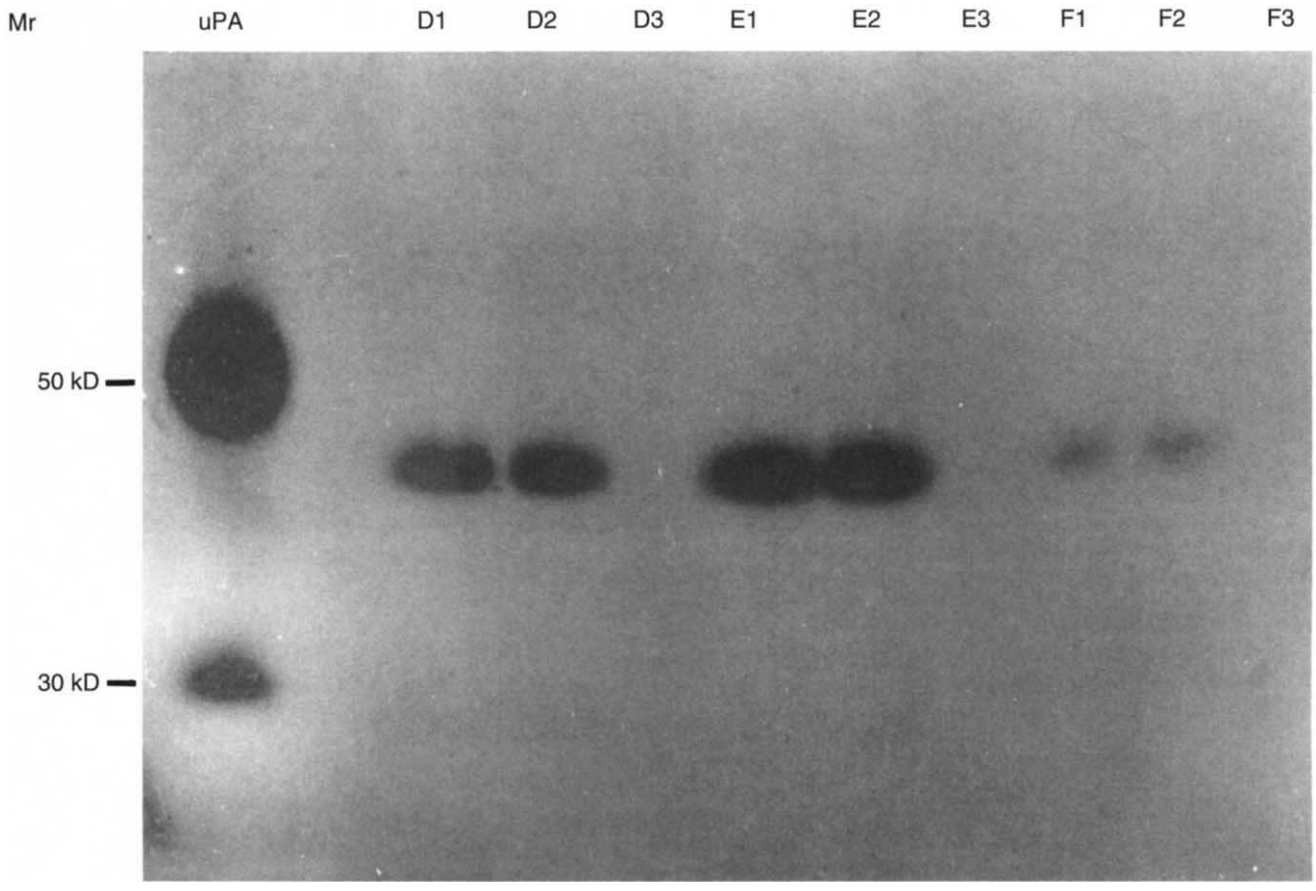

Fig. 2. *Plasminogen activator activity demonstrated by fibrin autography in glomerular lysates (preabsorbed with normal goat serum) from a normal rabbit (lane D1), a rabbit with heterologous phase anti-GBM GN (lane E1), and a rabbit with autologous phase anti-GBM GN (lane F1).* The same lysates were preabsorbed with anti-tPA antibody (lanes D2, E2, F2) or anti-uPA antibody (lanes D3, E3, F3). Human uPA was run in the left hand lane. Molecular weight calibrations are indicated.

Table 2. Tissue type plasminogen activator (tPA) activity and antigen in glomerular supernatants and lysates of normal rabbits and rabbits with anti-GBM GN

	tPA activity *IU/10⁵ glom*		tPA antigen *ng/10⁵ glom*	
	Supernatant	Lysate	Supernatant	Lysate
Normal	8.7 ± 3.7	UD	69.4 ± 22.4	2.9 ± 1.9
Fibrin associated GN	0.7 ± 0.5[a]	UD	17.7 ± 11.0[b]	1.7 ± 0.9
Fibrin independent GN	12.9 ± 8.7	UD	99.0 ± 22.3[c]	1.5 ± 0.9

UD is undetectable.
[a] $P < 0.08$ c.f. Normal
[b] $P < 0.06$ c.f. Normal
[c] $P < 0.08$ c.f. Normal

Table 3. Urokinase (uPA) activity in glomerular supernatants and lysates from normal rabbits and rabbits with anti-GBM GN

	uPA activity *IU/10⁵ glom*	
	Supernatant	Lysate
Normal	6.7 ± 1.3	132 ± 14
Fibrin associated GN	6.5 ± 1.8	95 ± 12
Fibrin independent GN	16.8 ± 1.2[a]	115 ± 0.9

[a] $P < 0.002$ c.f. Normal

glomerular fibrin deposition, crescent formation and the intensity of glomerular macrophage infiltration has also been demonstrated in this immune complex model of GN [17].

It is possible that the changes in GFA may be a response to glomerular inflammation unrelated to macrophage infiltraion. To address this question, heterologous phase anti-GBM GN, in which glomerular injury is independent of macrophages, was studied. Injury has previously been demonstrated to be dependent on complement-induced neutrophil accumulation [33] without significant glomerular fibrin deposition [10]. In this model, the direction of change of GFA was opposite to that seen with fibrin dependent GN, suggesting these changes are not merely related to severity or duration of injury. Thus, the changes in GFA are not uniform in all types of GN but may vary according to the particular mediator systems involved. The changes in glomerular fibrinolytic molecules observed in association with macrophages and fibrin deposition in this model of GN may indicate a causal relationship.

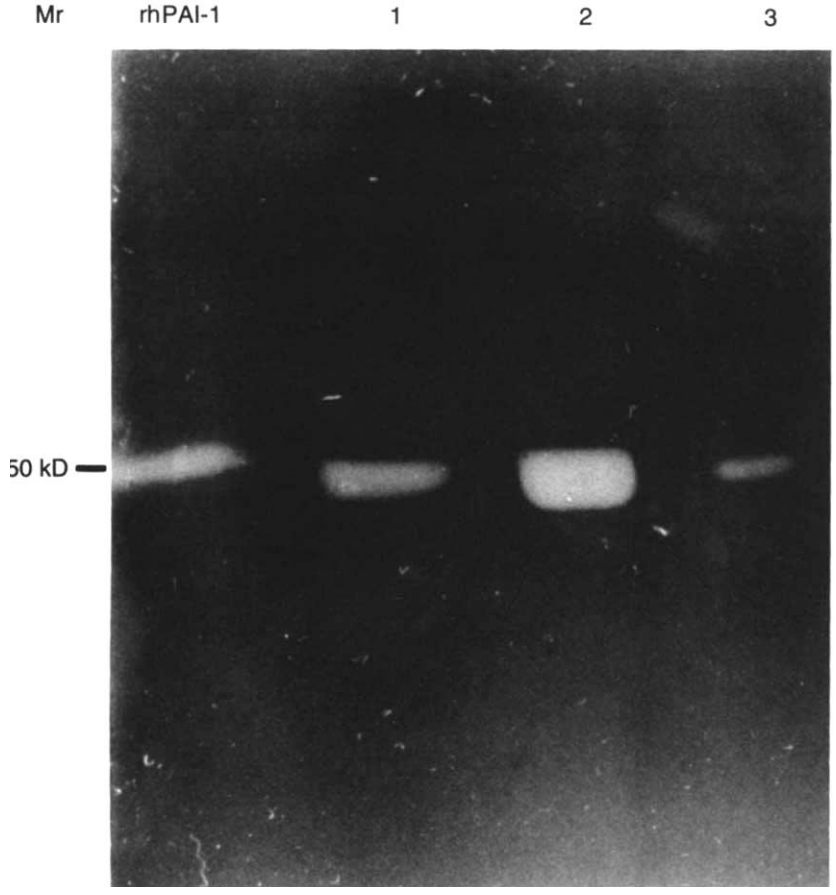

Fig. 3. *Plasminogen activator inhibitor activity demonstrated by reverse fibrin autography in glomerular supernatants from a normal rabbit (lane 1), a rabbit with autologous phase anti-GBM GN (lane 2) and a rabbit with heterologous phase anti-GBM GN (lane 3).* The left hand lane contains 50 kD recombinant human plasminogen activator inhibitor type 1 (rhPAI-1).

Table 4. Plasminogen activator inhibitor (PAI) activity in glomerular supernatants and lysates of normal rabbits and rabbits with anti-GBM GN

	PAI activity $aU/10^5$ *glom*	
	Supernatant	Lysate
Normal	9.6 ± 4.4	UD
Fibrin associated GN	42.4 ± 11.2[a]	UD
Fibrin independent GN	0.8 ± 0.8[b]	UD

UD is undetectable.
[a] $P < 0.03$ c.f. Normal
[b] $P < 0.003$ c.f. Fibrin dependent GN

The mechanisms by which macrophages induce changes in GFA are unknown. Macrophages have the capacity to produce plasminogen activator inhibitors [34], and therefore may contribute directly to the increase in PAI-1 production in glomeruli. Alternatively, macrophages may alter the expression of fibrinolytic molecules by intrinsic glomerular cells. Mesangial cells produce tPA and PAI-1 [35], and glomerular epithelial cells produce tPA, uPA and PAI-1 [36, 37] as do vascular endothelial cells [38, 39]. In addition, macrophage cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) can modulate the fibrinolytic activity of endothelial cells in a manner favoring fibrin deposition [40]. These cytokines have also been shown to alter the production of fibrinolytic molecules by glomerular epithelial cells [36]. Moreover, production of IL-1 and TNF by glomerular macrophages has been previously demonstrated in anti-GBM GN in rabbits [41, 42]. Thrombin also has the potential to alter the expression of fibrinolytic molecules by endothelial cells [43], mesangial cells [35] and glomerular epithelial cells [37]. Thus, the capacity of macrophages to activate the coagulation cascade generating thrombin provides a further mechanism by which macrophages may alter GFA.

In summary, down-regulation of GFA is associated with fibrin deposition and macrophage infiltration in anti-GBM GN in rabbits. Both reduced tPA expression and increased PAI-1 contribute to down-regulation of GFA. Together with augmentation of procoagulant activity, down-regulation of GFA appears to promote fibrin accumulation in this macrophage dependent model of proliferative GN.

Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia (NH&MRC) and the Australian Kidney Foundation and the Baxter Health Care Corporation. Mr. Malliaros is a recipient of an Australian Postgraduate Research Award. Dr. Tipping is an NH&MRC Research Fellow. Recombinant human PAI-1 was generously donated by Dr. Paul DeClerk, Centre for Thrombosis and Vascular Research, Leuven, Belgium, and the monoclonal anti-rabbit macrophage antibody (RAM-11) by Dr. A. Gown, Dept. of Pathology, University of Washington Medical Centre, Seattle, Washington, USA.

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